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Rodolfo R. Llinas

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Four areas of research were addressed this summer. The first related to the study of the miniature synaptic potentials in squid synapse. The necessary low noise microelectrode amplifiers and computer programs were developed which allowed us to do on-line analysis of the spontaneous miniature release. Using this paradigm, the second portion of the research related to the effects of Synapsin I and CAM kinase II in spontaneous and evoked transmitter release. The results supported our previous hypothesis that synapsin I functions as a vesicular caging molecule. The other two areas of research developed this summer had to do with discovering that the American funnel-web spider venom (FTX) can block calcium entry into the pre-synaptic transmission in squid giant synapse. This blockage is accomplished by the flow of synaptic transmission without affecting the ionic conductance that generate the action currents pre- and post-synaptically. Finally, using FTX, the calcium channel from squid CNS were isolated and reconstitute into lipid bilayer and shown to have similar voltage dependent currents as those measured with pre-synaptic voltage clamp in the giant synapse.

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22a. NAME OF RESPONSIBLE INDIVIDUAL

Dr. William O. Berry

22b. TELEPHONE (Include Area Code)

(202) 767-5021

22c. OFFICE SYMBOL

NL

Report to Dr. William Berry
on Research done in the Summer of 1988
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Miniature potentials in the squid giant synapse.

The miniature synaptic potentials recorded from the squid giant synapse have a nearly symmetrical waveform. Typically, it reaches peak amplitude in about 1 msec. and decays in 5-6 msec. The time course can be approximated by a difference between two exponential functions, with a rising and a decay time constant, i.e. $F(t) = a[e^{-t/t_d} - e^{-t/t_r}]$. Synaptic noise simulated on the basis of this observation was then used to test the resolution of spectral analysis. Briefly, a fast Fourier transform was performed and a best fit of the spectra was located by searching through a large number of time constant combinations. The results of the simulation showed that the calculated time constants recreated the original miniature potential waveform satisfactorily as long as the standard deviation of the background noise was less than twice that of the miniature amplitude. Thus, only those experiments with a background noise level lower than $200\mu V^2$ were selected for spectral analysis, i.e., with a standard deviation slightly larger than the minipotential amplitudes which have been estimated to be about $10\mu V$. This procedure was applied to experimental data (Fig. 1A), where different levels of noise were evoked by presynaptic depolarizations, and the frequency characteristics of these spectra provided consistent time constants for each synapse.

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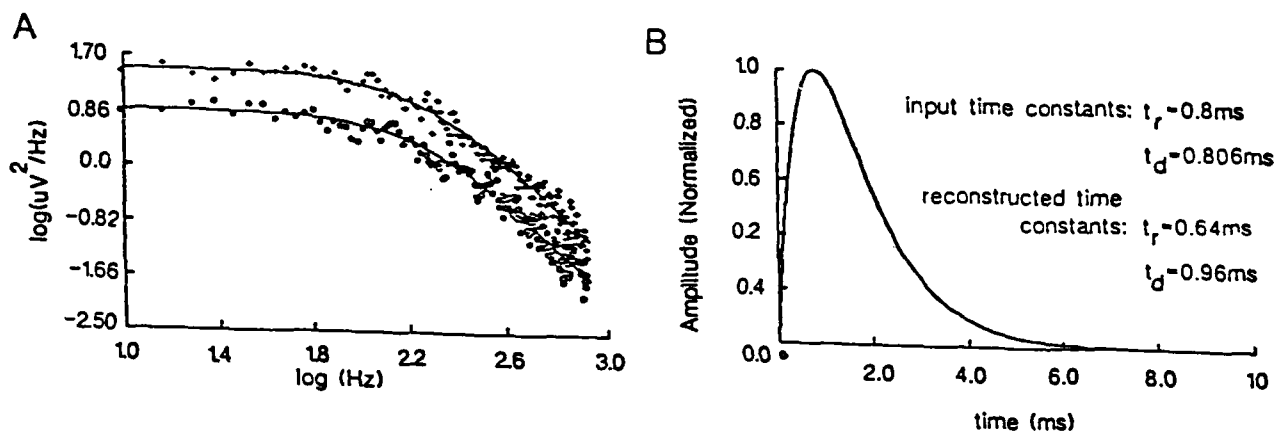


Figure 1A. Frequency spectrum for noise at two different miniature frequencies of 10,000 for the crosses, and 3,000 for circles. In B, time course of the reconstructed miniature potentials for these two noise levels.

In one example, rise time constants of 0.2-0.3msec and decay time constants of 1.5-1.6msec were obtained from recordings where there was a seven-fold difference in transmitter release (Fig. 1B). Similar spectral consistency was obtained before and after FTX application, which provided an additional support for the presynaptic calcium conductance blockage role of this toxin.

Action of Synapsin I and CAM kinase II on evoked and spontaneous miniature release in the squid giant synapse.

Synapsin I, in its dephospho- and its phospho- form, and CAM kinase II were injected into the presynaptic terminal digits of the squid giant synapse. The location of these proteins was monitored with fluorescent microscopy. The effects of these injections on spontaneous transmitter release were studied using the technique described above. The results indicated (Fig. 2) that dephosphosynapsin I reduced spontaneous and evoked quantal release in a manner which correlated temporally with its diffusion into the preterminal.



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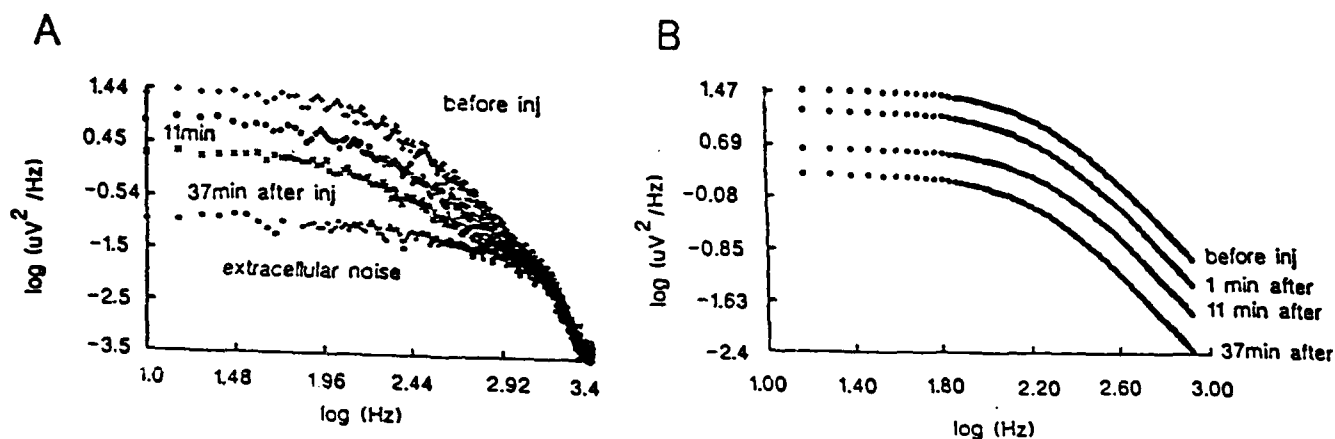


Figure 2. Frequency spectrum of spontaneous release as measured postsynaptically prior to injection (uppermost plot in A) and 11 and 35 mins after injection of Synapsin I (open circles and Xs). Frequency spectrum is then compared with the intracellular recordings (asterisks). In B, theoretical reconstruction of plots in A showing the frequencies before injection and 11 and 25 mins after injection of Synapsin I.

Phosphosynapsin I showed no effect. By contrast, CAM kinase II did not modify spontaneous release if the presynaptic potential was negative to -70mV . However, the low level of transmitter release produced by one second depolarizing pulses of the preterminal was increased by as much as 300% after the CAM kinase II injection. This indicated that a calcium entry, beyond that produced by the resting calcium conductance, is required for the injected CAM kinase II to increase transmitter release. The above results are consistent with the previous suggestion that these proteins control the availability of releasable vesicles by regulating the amount of vesicular caging by synapsin I whose binding to vesicles and to actin is phosphorylation-dependent

Blockage of calcium current by FTX A toxin fraction isolated from American funnel-web spider venom (FTX), has been shown to block calcium currents in central neurons. This fraction has been recently shown to have a low molecular weight (200 to 300 dalton). When tested on transmission at the squid giant synapse, the toxin was shown to block synaptic transmission without affecting either sodium or potassium voltage-dependent conductances. The blockage occurred without affecting the pre- or post-fiber action potentials.

Figure 3

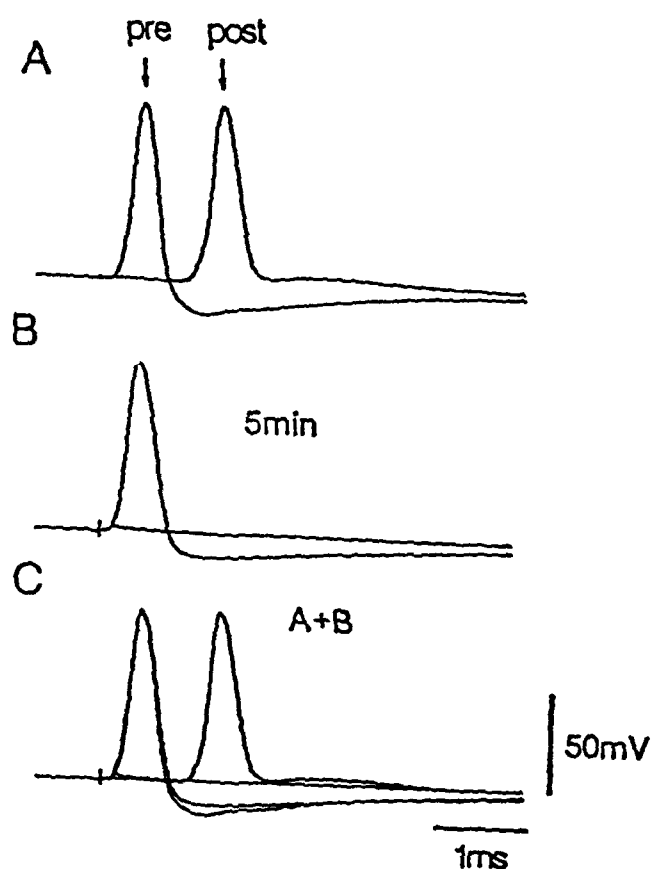


Figure 4

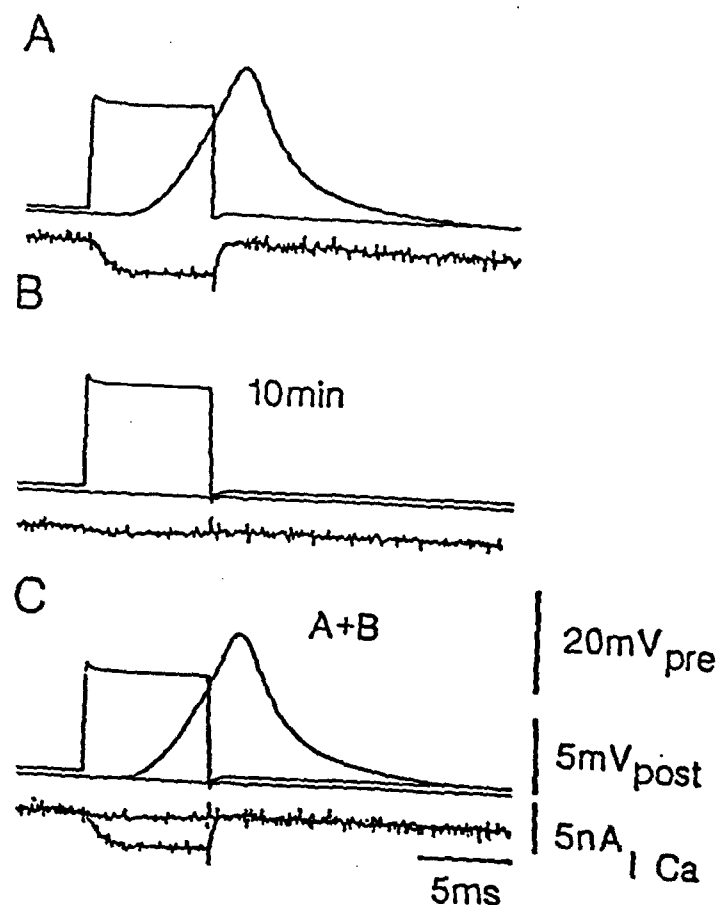


Figure 3A. Pre- and postsynaptic action potentials recorded in squid giant synapse. B, 5 mins after application of FTX. Note the total blockage of synaptic transmission and the small effect on the presynaptic action potential. In C, A and B are superimposed. Note that only a small change in the amplitude of the afterhyperpolarization is observed.

Voltage-clamp experiments of the presynaptic terminal show that the inward calcium current is blocked at submicromolar concentrations by FTX within a period of 5 to 10 minutes after direct application to the bath.

Figure 4A. Presynaptic voltage-clamp showing the presynaptic voltage steps (upper trace). Postsynaptic response and inward calcium current (lower trace). In B, following FTX total blockage of calcium entry and of postsynaptic transmitter release as seen by the lack of postsynaptic response. In C, A and B are superimposed.

The toxin was shown to be very slowly reversible, and its blockage to be related in a competitive way to $[Ca^{2+}]$. Finally, pressure injection of glutamate in the area of the postsynaptic fiber demonstrated that FTX had no effect on the post synaptic potential generated thereby indicating no effect of FTX on glutamate dependent postsynaptic channels.

Isolation of a voltage dependent calcium channel

FTX, a low molecular weight factor purified from American funnel-web spiders, which specifically blocks the squid presynaptic calcium current was used to construct an affinity chromatography gel. Squid optic lobe homogenate was solubilized, reacted batchwise with the gel and the bound protein eluted and reconstituted into lipid vesicles by sonication/dialysis. These vesicles were preloaded with the Quin-2. Addition of Ca^{++} to the external medium produced a rapid, sustained increase in the fluorescence, not seen in control vesicles. Influx was blocked by 50 μM Cd^{++} . FTX blocked in a dose-dependent and competitive manner with external Ca^{++} . When a Nernst potential was established by valinomycin, the Ca^{++} influx into the vesicles was found to be voltage dependent. Vesicles were also fused with lipid bilayers formed across the tip of a patch-clamp micropipette. Two types of channel-like activity were found. The first was characterized by voltage-dependent openings of 1 - 3 msec duration (mean). The opening probability, which was also voltage dependent, reached a maximum of 0.35 at a potential of 0 mV.

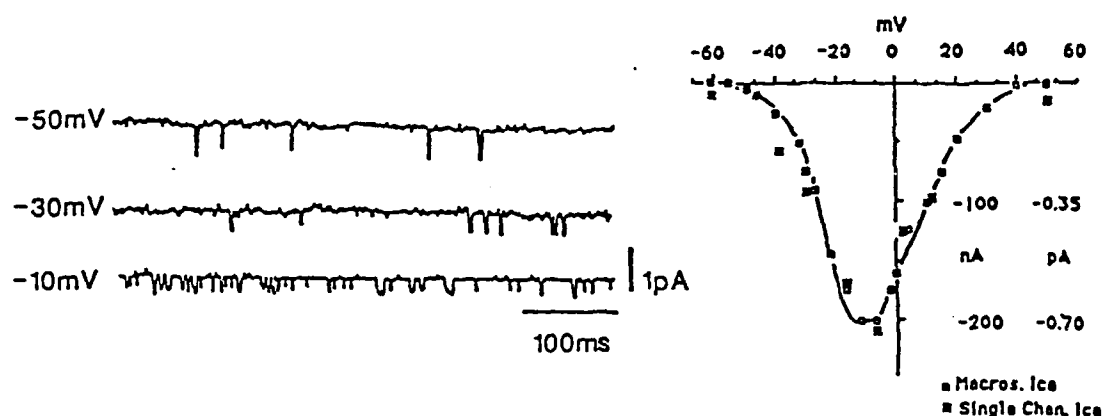


Figure 5. Single channel recordings from calcium channel incorporated into lipid bilayers showing increase in frequency of occurrence and diminution of current size as membrane is depolarized. B, reconstruction of current-voltage relation in single channel recording and comparison with macroscopic currents obtained in squid synapse.

The conductance was 15-20 pS in 80 mM Ba^{++} and 5-8 pS in 100 mM Ca^{++} . Comparison of the macroscopic currents obtained by summing multiple pulses reproduced closely the macroscopic Ica in squid terminal. When the cytoplasmic face of the protein was exposed to high concentrations of Ba^{++} , extremely long mean open times (300 msec) were observed having a similar conductance. In symmetric Ba^{++} solutions, replacement of the cytoplasmic solution with Cs^{+} resulted in conversion of long openings to short openings. High internal calcium (100 mM) did not change the opening-time mode. We conclude that using an affinity gel based on FTX, it has been possible to isolate and partially purify, a calcium channel with the properties expected for the presynaptic channel.

List of Publications

- Cherksey, B., Sugimori, M., Lin, J.W. and Llinás, R. Isolation of voltage dependent calcium channel from the squid central nervous system. (In press, Biol. Bull.)
- Sugimori, M., Lin, J.W., Cherksey, E. and Llinás, R. Blockage of calcium current by a factor from spider toxins. (In press, Biol. Bull.)
- Llinás, R., Sugimori, M., Lin, J.W., McGuinness, T.L. and Greengard, P. Effect of Synapsin I and CAM kinase II on evoked and spontaneous transmitter release in the squid giant synapse. (In press, Biol. Bull.)
- Lin, J.W., Sugimori, M. and Llinás, R. The analysis of miniature synaptic potentials in the squid giant synapse. (In press, Biol. Bull.)

List of professional personnel

Rodolfo R. Llinás, Principal Investigator
Mutsuyuki Sugimori, Investigator
Jen-Wei Lin, Investigator

Supporting staff: Alfred Benedek, Electronics Design Specialist
Matthew Canady, Programming Specialist

Collaborators: Paul Greengard (Rockefeller University)
Scott Brady (University of Texas, Dallas)